

FORM PTO-1390 (Modified)
(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

062308.0224

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)

CONCERNING A FILING UNDER 35 U.S.C. 371

Not Yet Known

09/529021

INTERNATIONAL APPLICATION NO.

PCT/US98/15835

INTERNATIONAL FILING DATE

31 July 1998

PRIORITY DATE CLAIMED

01 August 1997

TITLE OF INVENTION

DHA-CONTAINING NUTRITIONAL COMPOSITIONS AND METHODS FOR THEIR PRODUCTION

APPLICANT(S) FOR DO/EO/US

Raymond M. GLADUE et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4))
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Executed Verified Statement Claiming Small Entity Status as a Small Business Concern

09/529021

U.S. APPLICATION NO. (IF KNOWN) SEE 37 CFR 1.5) Not Yet Known		INTERNATIONAL APPLICATION NO. PCT/US98/15835		ATTORNEY'S DOCKET NUMBER 062308.0224	
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21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			CALCULATIONS PTO USE ONLY		
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	\$970.00				
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	\$840.00				
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	\$690.00				
<input checked="" type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	\$670.00				
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	\$96.00				
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$670.00			
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).			\$0.00		
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	x \$18.00	\$72.00	
Independent claims	5 - 3 =	2	x \$78.00	\$156.00	
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>			\$0.00		
TOTAL OF ABOVE CALCULATIONS =				\$898.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <input checked="" type="checkbox"/>			\$449.00		
SUBTOTAL =				\$449.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			\$0.00		
TOTAL NATIONAL FEE =				\$449.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>			\$0.00		
TOTAL FEES ENCLOSED =				\$449.00	
				Amount to be: refunded	\$
				charged	\$

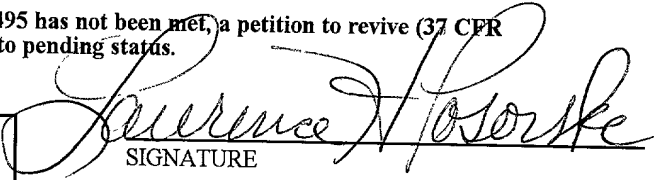
☒ A check in the amount of **\$449.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **02-0375** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Laurence H. Posorske Baker Botts L.L.P. The Warner; Suite 1300 1299 Pennsylvania Avenue, N.W. Washington, DC 20004-2400	 SIGNATURE Laurence H. Posorske NAME 34.698 REGISTRATION NUMBER April 6, 2000 DATE
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**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(d) AND 1.27 (c)) - SMALL BUSINESS CONCERN**Docket No.
062308.0224Serial No.
Not Yet KnownFiling Date
Concurrently HerewithPatent No.
N/AIssue Date
N/AApplicant/ **Raymond M. GLADUE et al.**
Patentee:Invention: **DHA-CONTAINING NUTRITIONAL COMPOSITIONS AND METHODS FOR THEIR PRODUCTION**

I hereby declare that I am:

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Martek Biosciences CorporationADDRESS OF CONCERN: 6480 Dobbins Road, Columbia, Maryland 21045

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above identified invention described in:

- ☐ the specification filed herewith with title as listed above.
☒ the application identified above.
☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☒ no such person, concern or organization exists.
☐ each such person, concern or organization is listed below.

FULL NAME N/A

ADDRESS _____

☐

Individual

☐

Small Business Concern

☐

Nonprofit Organization

FULL NAME N/A

ADDRESS _____

☐

Individual

☐

Small Business Concern

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Nonprofit Organization

FULL NAME N/A

ADDRESS _____

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Individual

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Small Business Concern

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Nonprofit Organization

FULL NAME N/A

ADDRESS _____

☐

Individual

☐

Small Business Concern

☐

Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING:

Steve Dubin

TITLE OF PERSON SIGNING _____

OTHER THAN OWNER:

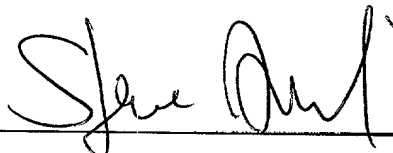
Secretary

ADDRESS OF PERSON SIGNING:

6480 Dobbin Road

Columbia, MD 21045

SIGNATURE: _____



DATE: _____

4/5/00

DHA-CONTAINING NUTRITIONAL COMPOSITIONS AND METHODS FOR THEIR PRODUCTION

5 BACKGROUND OF THE INVENTION

The last decade has witnessed a decline in the resources of natural fisheries that has now reached a level of global environmental crisis. Thirteen of the worlds seventeen major fisheries are classified by the United Nations Food and Agriculture Organization (F.A.O.) to be in peril or in steep decline. Even in the best case scenario of both
10 maintaining the fishery catch at a record 100 million metric tons and conservative population growth, there will be a shortfall in global seafood supply by the end of this decade for the first time in human history. Furthermore, per capita global seafood consumption is predicted to increase as the worlds population shifts during the next two decades from industrialized countries to todays undeveloped and emerging economies;
15 traditionally large consumers of seafood. The only way to meet the global demand for seafood by the year 2000 is if aquaculture replaces the insufficient natural fisheries catch.

Expansion of the aquaculture industry requires that several significant problems be addressed, and one of the most significant hurdles to establishing and maintaining an economically viable aquaculture operation is the difficulty of supplying nutritionally
20 balanced feeds. Larval fish, bivalves and crustaceans raised in the wild consume a mixed population of feed organisms that collectively provide balanced nutrition. On the other hand, fish larvae, bivalves and crustaceans raised in aquaculture farms can be difficult to rear and require live feeds (algae or algae-fed rotifers and Artemia) for their nutrition. These live feeds are difficult to produce and maintain, require high labor inputs and
25 specialized facilities, and as a result larval feeds constitute a significant cost to the aquaculture industry.

It is important that aquaculture feeds be nutritionally balanced so that the larvae receive proper nutrition. DHA (docosaheaxaenoic acid) has been identified as an important nutrient that contributes significantly to larval growth and survival (Fulks, W
30 and KL Main (eds). 1991. "Rotifer and Microalgae Culture Systems. Proceedings of a US-Asia Workshop." Honolulu, HI. The Oceanic Institute. Tamaru, CS, CS Lee and H Ako, 1991. "Improving the larval rearing of striped mullet (*Mugil cephalus*) by manipulating quantity and quality of the rotifer, *Brachionus plicatilis*." In: W. Fulks and

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KL Main (eds), 1991). Larvae ultimately acquire these fatty acids from algae, either by directly feeding on algae with high levels of polyunsaturated fatty acids or by feeding on rotifers and Artemia that have been reared on algae high in polyunsaturated fatty acids. Unfortunately, the algae, artemia and rotifers used at aquaculture farms are low in DHA, reducing the survival rates for the larvae below their maximal rate and increasing the cost of the final aquaculture farm product. If sufficient DHA could be provided to the larvae it is expected that the survival rate for larvae would increase, thus reducing the cost of farm-raised seafood.

SUMMARY OF THE INVENTION

10 In one embodiment, this invention provides a particulate material suitable for use as an aquaculture feed. The particulate material has a high proportion of DHA residues in the lipid fraction, which may be up to 35% of the material. Preferably, the material has a mean particle size of from about 5 microns to about 10 microns.

15 In another embodiment, this invention provides a method for preparing a particulate material suitable for use as an aquaculture feed from microbial biomass, preferably from algal cells having a high content of DHA residues, by obtaining a lipid fraction from the biomass, preferably by solvent extraction of broken cells, followed by separating a fraction containing phospholipids and proteins from the lipid fraction, and removing water from the protein/phospholipid fraction to form a low moisture particulate, preferably by spray-drying the protein/phospholipid fraction.

20 In still another embodiment, this invention provides a particulate material containing phospholipids having docosahexaenoic acid (DHA) residues, the particulate material being prepared by drying a slurry comprising a polar lipid extract from DHA-containing microbes, and dry, particulate material may be prepared from a slurry which is substantially free of material that did not come from the DHA-containing microbes, particularly by spray drying the slurry. Typically, at least two thirds of the dry matter in the polar lipid extract of DHA-containing microbes is material derived from the microbial cells; preferably less than 25% of the dry matter is non-microbial, more preferably less than 20%, even more preferably 15% or less. Typically, the spray dried particulate has number average particle size between 5 microns and 10 microns.

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In yet another embodiment, this invention provides a method for preparing a DHA-containing particulate material comprising (a) lysing DHA-containing microbial cells; (b) extracting lysed cells with solvent; (c) separating a polar lipid fraction from the extract; and (d) drying the polar lipid fraction, with or without addition of other
5 nutrients, to form a particulate material. Preferably, the polar lipid fraction is an aqueous slurry which is dried by spray drying.

In a particular embodiment, this invention provides a method for preparing a DHA-containing particulate material comprising drying a slurry containing polar lipids extracted from dinoflagellates, wherein the dried material is in the form of particles
10 having a mean particle diameter between 5 and 10 microns.

In yet another embodiment, this invention provides an aqueous emulsion or suspension containing phospholipids with DHA residues obtained from a polar lipid extract from DHA-containing microbes. Preferably, at least 10 % of the fatty acid residues in lipids of the DHA-containing microbes are DHA residues. More preferably,
15 at least 10 % of the fatty acid residues in polar lipids of the DHA-containing microbes are DHA residues.

In still another embodiment, this invention provides a composition comprising a particulate material containing phospholipids with DHA prepared by drying a slurry comprising (a) a polar lipid extract from DHA containing microbes and (b) a meal
20 containing protein and/or carbohydrate. Preferably, the meal comprises microbial cells or cell fragments, which may be cells or cell fragments which have been extracted to remove part of the lipids, or even most of the lipids. Preferred microbial cells or cell fragments are from *Chlorella*, *Cryptothecodinium*, or a yeast such as *Saccharomyces*, or a fungus, such as *Mortierella*, *Schizochytrium*, or *Thraustochytrium*.

25

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Producing high DHA-containing oil from biomass requires several different processing and refining steps. Each refining step produces waste fractions as a byproduct of the oil purification. These byproducts contain significant levels of DHA

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and other nutrients that can have potential use as aquaculture feeds for fish larvae, bivalves and crustaceans.

One byproduct in particular contains a very high level of DHA as well as other nutrients (carbohydrate, protein, etc) that are useful and valuable in larval nutrition. This byproduct, hereinafter called "DHA Phospholipids," also contains significant levels of all of the amino acids that are considered essential for larvae. The ten amino acids that are generally recognized to be essential for most fish species are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, see, e.g., Shepherd, Jonathan and Niall Bromage, *Intensive Fish Farming*, Chapter 5, Nutrition and Growth, Blackwell Scientific Publications, Boston, 1992, pp. 154-197. The DHA Phospholipid material contains all of these amino acids at levels comparable to the levels found in some other aquaculture feeds. The DHA Phospholipid material contains phospholipids having a high proportion of DHA residues, as well as other lipid components such as triglycerides, free fatty acids, sterols, glycolipids, etc. The biochemical composition of the DHA Phospholipids, obtained as spray-dried particles, is given in Table 1. The fatty acid composition of the particulate material is given in Table 2.

Source of DHA-containing Material

The principal source of DHA in the biosphere is from algae. Numerous algal species produce DHA. One species in particular (*Crypthecodinium cohnii*) has been manipulated to produce very high levels of DHA. This organism has been cultivated at large scale and the biomass has been used for the production of a high DHA-containing oil. Suitable processes for producing DHA-containing biomass from *C. cohnii* are provided in U.S. Patent Nos. 5,397,591 and 5,492,938, incorporated herein by reference.

Similar DHA Phospholipid material may be made from any single celled organism that contains a significant amount of DHA. This would include various oleaginous fungi, various algae (especially members of the class Dinophyceae, Bacillariophyceae, Chlorophyceae, Prymnesiophyceae and Euglenophyceae), as well as organisms of uncertain taxonomic status such as *Thraustochytrium* or *Schizochytrium*. Suitable

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processes for producing DHA-containing biomass from *Thraustochytrium* or *Schizochytrium* are provided in U.S. Patent No. 5,130,242, incorporated herein by reference.

Typically, microbes serving as a source of DHA Phospholipids according to this invention will have at least 5% of the fatty acids and fatty acid residues in the microbe as DHA, more typically at least 10%, and preferably at least 20%. Preferably, at least 10% of the fatty acid residues in the polar lipids of the source microbe will be DHA, more preferably at least 20% of the residues will be DHA.

Preparation Methods

DHA Phospholipids may be obtained from a lipid fraction of the DHA-containing biomass. A preferred method for obtaining a suitable lipid fraction is solvent extraction of lysed cells. In a preferred mode, the biomass may be spray-dried, which will lyse most cells, and the spray-dried material extracted with a solvent such as hexane. Grinding or homogenizing the solvent-biomass mixture may facilitate extraction of the lipid fraction (oil) and may also promote recovery of non-lipid components that are desirable for the aquaculture feed. The DHA Phospholipid material is separated from the oil as a water/salt-containing precipitate. Suitable procedures for such separation are readily adapted from analogous processes in vegetable oil refining.

Although the DHA Phospholipid byproduct contains useful levels of DHA and other nutrients, a suitable method must be found to provide this material to larvae. Many fish larvae require live feed, and bivalves and crustaceans also require either live feed organisms or particles of a limited size range. Thus, further processing is preferable to merely providing this material directly to the fish larvae, bivalves or crustaceans.

For those organisms requiring a live feed, the DHA Phospholipids can be fed to artemia and/or rotifers (both natural food organisms for larvae), with the goal of raising the DHA level of the artemia/rotifers and subsequently raising the DHA level of the larvae. For those organisms that are filter feeders, the material may be supplied as small particles and thus provide a direct enrichment of DHA into the diet.

In order to prepare particles of the proper size for ingestion by artemia, rotifers and filter feeders, the DHA Phospholipid material is dried by any suitable process, such

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as spray drying, granulation, encapsulation and the like. When DHA Phospholipid material was freeze dried, the resultant particles were too large to be useful. The high fat content in the freeze-dried material did not permit the particle size to be reduced by grinding. Spray drying yielded a crumbly mass consisting of particles that were of an appropriate size range for consumption by artemia, rotifers and filter feeders. Mean particle size was determined to be about 5-10 microns in diameter; the distribution of partial sizes about the mean was quite broad (at least from >30 microns to <1 micron). Although the material was subjected to heat during the drying process, the DHA level was not appreciably reduced by the temperature of the dryer. Thus, spray drying yielded a product with a high DHA content and an appropriate particle size.

Preferably, the DHA level of the spray dried material should be maximized, thus making the material an even more valuable source of DHA for larvae. Typically, the DHA level would be raised by increasing the amount of DHA-containing oil in the DHA Phospholipid material, which may be accomplished by adding refined triglyceride oil, partially purified triglycerides or crude lipid extract from a microorganism that is high in DHA. The inventor has noted that, when the triglyceride level (measured as esterified fatty acid level) exceeded about 30% of the total dry weight of the DHA Phospholipid, the material did not spray dry as well and appeared to form very large particles that were less suitable for this application. At triglyceride contents above 40%, the final spray-dried product is very sticky due to the high fat content, which may explain the apparent high particle size. This material is not as easy to handle as the lower triglyceride material, but it still can be used as an artemia and rotifer enrichment product. Of course, supplemental DHA may also be supplied from non-microbial sources, including purified DHA-alkanol esters, but these sources may be less economical.

While DHA Phospholipid may be spray dried directly, spray drying may be facilitated if the slurry contains typical spray drying agents when it is fed into the spray dryer. Suitable spray drying carriers include maltodextrines, starch, gelatin, sugars, and molasses. Preferred carriers include sodium alginate and gum arabic. In addition, various additives whose presence in the final particulate is desirable for nutritional reasons may also be added to the slurry before drying, such as proteins, carbohydrates,

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fatty acids and lipids, or vitamin sources, including additives like yeast extract, starch, gelatin, gluten, etc. In addition, amino acids may be added to the slurry, such as methionine, a particularly important amino acid in fish nutrition. Alternatively, algal biomass may be added to the slurry as a spray drying aid, preferably using algae which are naturally food for rotifers or fish larvae.

In one embodiment, particulate material may be prepared by formulating and spray drying the DHA Phospholipids with microbial biomass, which may be whole or lysed cells or cellular material after lipid extraction. DHA Phospholipids may be mixed with biomass in any ratio, including 1:100 to 100:1, preferably in ratios from 1:10 to 10:1. The mixture is typically spray dried from an aqueous slurry to form a material with the appearance or consistency of cornmeal. Typically, the biomass will be from microbes that have known nutritional uses, such as *Chlorella*. *Chlorella* is consumed as a human food product, and DHA is an important human nutrient, so such particulate material is also useful in human nutrition. Of course particulate material produced according to this invention may be used to supply DHA in human nutrition whether or not the particulate contains *Chlorella*, so long as the process conditions and any other additives included in the slurry are suitable for human consumption.

While the DHA Phospholipid is particularly suited to spray drying, particulate material containing high DHA levels may also be prepared according to this invention by granulation or encapsulation. Conditions for granulation or encapsulation of DHA Phospholipid may be readily selected by the skilled worker, typically using one or more of the additives which are listed above for use in spray drying, such as alginate or gum acacia. Of course, DHA Phospholipids may also be coated on oilseed meal (e.g., soybean meal, cotton seed meal, corn germ, etc.). Furthermore, mixtures of DHA Phospholipids with solvent extracted algal biomass ("biomeal") are also suitable for freeze-drying. While this invention is particularly suitable for producing dry products enriched in DHA, the DHA Phospholipids obtained from microbes according to this invention may be formulated in a suspension or emulsion for subsequent use, particularly as a nutritional supplement. Such emulsions or suspensions containing polar lipids may be routinely prepared, as is known in the art.

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Particle size distribution in the dried material may be adjusted by fractionation in, e.g., cyclones or filter bags, to remove particles that are too large, but preferably the drying process will produce a particulate material of mean particle size from 5 microns to 10 microns. Typically the dried DHA Phospholipids will be mixed with sea water before feeding to larvae, rotifers, or artemesia. Varying the agitation in this mixing step will permit post-drying adjustment of the particle size.

Another fatty acid with significance in both aquaculture and human and animal nutrition is arachidonic acid (AA). Arachidonic acid may also be obtained in lipid extracts of microbes, although preferred sources of AA are different species from the preferred sources of DHA. Suitable microbial sources of AA and methods for growing them are taught, for example, in U.S. Patent No. 5,658,767. Extraction of crude lipids from AA-containing microbes leaves a protein-and-carbohydrate containing residue that may also still have appreciable amounts of AA as fatty acids and/or fatty acid residues. This material may be referred to as AA biomeal.

During the processing and refining of the AA-containing crude lipid extract, several fractions are produced, including AA Phospholipids. The AA Phospholipids can also be blended with the DHA Phospholipids to produce a material that contains both fatty acids in the ratio that will provide for optimal nutrition. Such a blend can be prepared as a dry material or as an emulsion or suspension. The AA Phospholipids can also be blended with the DHA biomeal and dried or emulsified to produce a material that is high in arachidonic acid. Through the use of different ratios of DHA Phospholipids, DHA biomeal, AA biomeal, and/or AA Phospholipids, a material can be produced that has the appropriate ratio of DHA and AA for optimal nutrition.

EXAMPLES

In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

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Example 1. Process for producing DHA Phospholipids

Crypthecodimium cohnii is grown by heterotrophic fermentation as described in U.S. Patent No. 5,407,957, incorporated herein by reference. At the conclusion of the fermentation, the DHA-rich biomass is concentrated by centrifugation to about 18% solids (range is 5-35% solids). The concentrated biomass is held in a chill tank (50-60 °F) prior to spray drying. The material is pumped under high pressure through a spray dryer with an inlet temperature ranging between 300 - 400 °F, but typically 350-380 °F. The flow rate into the spray dryer is adjusted as necessary to achieve an outlet temperature of 180-240 °F, but typically 205-215 °F. The dried biomass recovered from the spray dryer has a moisture content ranging from 1-15% (typically 6%).

The dried biomass is slurried with hexane at the ratio of about 2 liters of hexane per kilogram of dried biomass. The hexane/biomass mixture may be milled in a colloid mill to insure that all cells are broken and that maximum oil can be extracted. Alternatively, the biomass/hexane mixture may be homogenized (e.g., forced through an orifice with pressure drop of approximately 7-800 atm). The hexane fraction (containing the DHA-rich oil) is separated from the solids using a counter current extractor. The hexane exiting from the extractor has an oil content of about 5% (v:v).

The oil/hexane mixture is centrifuged to remove any fine particles that were not previously removed in the extractor. The hexane is partially removed by evaporation to produce a mixture of about 65% oil in hexane. This oil/hexane mixture is cooled to about 40 °F and held at this temperature for about 12 hours. The material that precipitates under the low temperature condition is removed by centrifugation. The remaining hexane associated with the oil is removed by evaporation to produce a hexane-free oil.

The oil is transferred to a refining tank where 50% caustic (0.74% w/w based on oil), 85% phosphoric acid (0.2% w/w based on oil), and oleic acid (to bring the final free fatty acid level to 5% in the oil) are added to the crude oil. The mixture is agitated and heated to approximately 70 °F for 15-30 minutes. The combination of heat and chemicals causes the DHA Phospholipids to precipitate out of the oil. The DHA Phospholipids are collected by centrifugation.

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The DHA Phospholipid fraction is homogenized to insure a uniform mixture. At this point the material can be used "as is" for spray drying or it can be blended with various nutrients and other compounds (i.e. vitamins, protein, alginates, antifoams, etc.) before proceeding with spray drying. The solids content of the DHA Phospholipids can range from 10% up to over 80%. Typically, the solids content is diluted to around 25-35% to form a pumpable slurry for spray-drying, although solids content in the slurry may range from 10-50%. The material is spray dried using either a high pressure injection nozzle or a rotary atomizer. Suitable inlet temperatures range between 170 - 240 °F, but typically around 200 °F, with the material being fed to the spray dryer at a rate sufficient to maintain an outlet temperature on the spray dryer of 150 - 200 °F, but typically between 180-190 °F. In an alternative mode, the slurry is spray dried with inlet temperature of about 300°C (540 °F) and outlet temperature of about 110 °C (230 °F). Routine optimization of drying conditions by adjustment of inlet temperature and flow rate in view of the guidelines provided herein is contemplated for this invention.

The DHA Phospholipid material collected from the spray dryer has a moisture content of about 4%, and the biochemical composition of three representative batches is given in Table 1, the fatty acid composition of the same 3 batches, plus a 4th is given in Table 2. These batches were all suitable for spray drying. If the esterified fatty acid levels exceed about 30-35%, then the material does not spray dry as well. Rather than forming discrete particles, it tends to form larger clumps, presumably due to the higher oil content in the material.

Example 2. DHA-containing Particulate Blends

The DHA Phospholipids may be formulated with other materials and nutrients. Table 3 compares spray dried DHA Phospholipids to the composition of DHA Phospholipids + Biomeal and DHA Phospholipids + *Chlorella* prepared as follows:

a. DHA-Phospholipids was mixed with biomeal consisting of dried biomass from Example 1 after extraction with solvent, and then spray dried as described in Example 1. The purpose of this blending was to reduce the total fatty acid content of the spray dried material so that it wouldn't be so sticky. This blend was certainly less sticky so it may be considered an improvement to the basic material, even though

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blending in the biomeal did reduce the total fat content compared to the basic DHA Phospholipids.

b. The DHA Phospholipids were blended with the alga *Chlorella*, and this mixture was spraydried as described in Example 1. *Chlorella* is a natural food source for rotifers and is high in protein. By blending with *Chlorella* it was possible to reduce the stickiness of the final spray dried DHA Phospholipids while at the same time providing nutrients from the natural food source. The initial trial was to blend 3:1 (w:w) DHA Phospholipids: *Chlorella*, and this produced a material that handled very well. A wide ratio of blends is possible. One variation is to have a high ratio of *Chlorella* to DHA Phospholipids. This would basically provide the natural marine food source with extra DHA, so the material could be as a regular, routine feed and not just as an enrichment to increase the DHA content of the rotifers prior to feeding them to fish larvae.

c. The DHA Phospholipids have also been mixed with a protein source for the purpose of improving the protein content (and therefore nutritional content) of the spray dried material. Specifically, the DHA Phospholipid was blended with soy protein hydrolyzate in a ratio of 3:1 (w:w) of DHA-Phospholipid: soy protein hydrolyzate. This demonstrates that the DHA Phospholipid can be mixed with other nutrients to make a particulate.

Example 3 Blends of DHA and Arachidonic Acid Materials

The DHA Phospholipids can be formulated with arachidonic acid to produce a material that provides both of these fatty acids in a single nutrition product.

Arachidonic acid-containing biomass is produced from *Mortierella* as outlined in U.S.

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Patent No. 5,658,767. The *Mortierella* biomass is harvested by centrifugation and dried under vacuum. The dried biomass is extracted with hexane to remove the arachidonic acid-containing lipids, and the crude lipid fraction is processed in a manner similar to that used in Example 1. The mass remaining after lipid extraction removal (referred to as AA

5 biomeal) contains a high level of protein but also significant amounts of arachidonic acid. This AA biomeal can be blended with the DHA Phospholipids to produce a higher protein material that also contains significant amounts of both arachidonic acid and DHA.

For purposes of clarity of understanding, the foregoing invention has been

10 described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are

15 apparent to persons of skill in edible oil extraction and processing, microbial fermentation, nutrition, medicine, pharmacology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains.

20 All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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Table 1. Biochemical composition of spray dried DHA Phospholipids

Component	Prep. 1	Prep. 2	Prep 3
Total Fatty Acid (% of total wt)	57.4	61.3	55.6
esterified fatty acid (% of total wt)	25.6 (48% DHA)	24.4 (47.7% DHA)	22.3 (47.9% DHA)
free fatty acid (% of total wt)	31.8 (19% DHA)	36.9 (22.5% DHA)	33.3 (25.0% DHA)
Sterol (% of total wt)			
Total Carbohydrate (% of total wt)	8.0	7.2	8.3
glucose	6.1	4.7	6.2
galactose	1.9	2.5	2.1
fucose	0.0	0.0	0.0
Protein (% of total wt)	13.2	9.7	11.7
Amino Acid (% of protein)			
methionine	2.0	4.3	2.23
cystine	0.8	1.1	0.75
lysine	4.3	3.7	4.92
phenylalanine	5.0	5.0	1.73
leucine	8.3	8.2	7.54
isoleucine	4.0	4.2	4.65
threonine	5.9	5.4	5.89
valine	7.2	5.4	6.99
histidine	2.5	2.2	2.56
arginine	4.8	4.3	4.74
glycine	6.2	6.2	7.05
aspartic acid	10.4	10.3	10.66
serine	5.5	6.0	6.26
glutamic acid	13.7	13.8	14.06
proline	6.2	4.7	6.77
hydroxyproline	0.5	1.7	0.00
alanine	7.6	8.0	8.81
tyrosine	3.8	4.3	2.94
tryptophan	1.4	1.2	1.46
Moisture (% of total wt)	4.4	4.00	6.0
Fiber (% of total wt)	0.7	1.0	0.6
Ash (% of total wt)	10.4	10.1	10.6
sodium (% of total wt)	2.6	not analyzed	3.38
TOTAL	94.1	93.3	92.7

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Table 2. Fatty acid composition of spray dried DHA Phospholipids (% of total fatty acid)

Fatty Acid	Prep. 1 esterified	Prep. 1 non- esterified	Prep. 2 esterified	Prep. 2 non- esterified	Prep. 3 esterified	Prep. 3 non- esterified	Prep. 4 esterified	Prep. 4 non- esterified
08:00					0.06		0.25	0.23
10:00	0.67	1.22	0.83	1.11	0.45	0.76	1.39	0.5
11:00								
12:00	4.34	2.69	4.36	2.65	3.34	3.14	5.71	2.71
13:00								
14:00	15.94	5.81	15.52	7.7	14.83	9.68	16.71	8
14:01	0.13				0.12		0.11	
16:00	17.2	11.77	16.21	13.61	16.45	15.26	20.8	16.34
16:01	1.7	0.47	1.63	0.6	1.4	0.53	1.16	0.46
16:02								
16:03								
17:00					0.13			
18:00	0.88	1.89	0.94	1.91	0.96	1.82	1.11	1.68
18:1n-9	10.21	46.29	12.82	40.61	13.29	36.1	9.22	36.97
18:1n-7		0.62		0.63		0.46		0.56
18:02	0.1	8.56		7.47	0.4	6.06	0.1	7.3
18:3n-6					0.27			
18:3n-3		0.12						0.11
18:04								
20:00		0.09		0.32	0.26			0.09
20:01		0.15						0.13
20:02								
20:3n-9								
20:3n-6				0.3	0.2			
20:3n-3								0.61
20:04								
20:05								
22:00								
22:01								
22:04						0.46		0.42
22:5n-6					0.64			
22:5n-3	0.53	0.28					0.28	
22:06	48.05	19.62	47.68	22.52	46.96	25.01	42.85	22.78
% Fatty acid in sample	25.64	31.83	24.4	36.9	22.34	33.26	31.2	45.63
% Unknown	0.23	0.41	0	0.26	0.23	0.71		1.09

COMPARISON OF AQUACULTURE PRODUCTS

TABLE 3A

Category	DHA Phospholipids	DHA-PL + C.cOHNI	DHA-PL + Chlorella
Total Fatty	76.8	57.3	56.7
Acid (%)			
DHA (% of fat)	30.9	32.91	30.54
DHA (%DW)	23.7	18.9	17.3
EPA (% of fat)	0.11	0	0
Carbohydrate	2.1	18.1	9.7
Protein (%)	11.7		10.2
Moisture (%)	1.5		2.4
Ash (%)	9.3		10.8
Fiber (%)	1		0.8
Total	102.4		97.2

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FATTY ACID ANALYSIS
TABLE 3B

Fatty Acid (% of total fatty acid)	DHA Phospholipids	DHA-PL + Ccohnii	DHA-PL + Chlorella
08:00	0.24	0.17	0.06
10:00	0.86	0.64	1.06
11:00			0.06
12:00	3.93	3.41	3.23
13:00		0.00	
14:00	11.54	12.05	8.61
14:01	0.04	0.06	0.05
16:00	18.15	16.58	12.49
16:01	0.74	0.96	1.18
16:02			0.12
16:03			0.08
17:00			
18:00	1.45	1.42	1.87
18:1n-9	25.7	26.93	33.88
18:1n-7	0.33	0.32	0.46
18:02	4.38	4.58	7.46
18:3n-6			
18:3n-3	0.07		0.30
18:04			
20:00	0.05		
20:01	0.08		
20:02			
20:3n-9			
20:3n-6			
20:3n-3	0.36		
20:04			
20:05			
22:00			
22:01			
22:04	0.25		
22:5n-6			
22:5n-3	0.11		
22:06	30.93	32.91	28.85
Total (% fat in fatty acids)	99.22	100.03	99.76
% Fatty acid in sample	76.8	57.30	56.70
% Unknown			

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CLAIMS:

1. A particulate material containing phospholipids with docosaheptaenoic acid (DHA) residues prepared by drying a slurry comprising a polar lipid extract from DHA-containing microbes.
- 5 2. The particulate material of claim 1, wherein the mean particle size is between 5 microns and 10 microns.
3. The particulate material of claim 1, wherein the slurry is dried by spray drying.
4. The particulate material of claim 1, wherein the slurry is substantially free
10 of material which did not originate in said DHA-containing microbes.
5. The particulate material of claim 1, wherein at least 10 % of the fatty acid residues in lipids of said microbes are DHA residues.
6. The particulate material of claim 1, wherein at least 10 % of the fatty acid residues in polar lipids of said microbes are DHA residues.
- 15 7. The particulate material of claim 1, wherein said microbes are dinoflagellates.
8. The particulate material of claim 1, wherein said microbe are *Cryptocodinium cohnii*.
9. A method for preparing a DHA-containing particulate material
20 comprising drying a slurry containing polar lipids extracted from dinoflagellates, wherein the dried material is in the form of particles having a mean particle diameter between 5 and 10 microns.

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10. A method for preparing a DHA-containing particulate material comprising lysing DHA-containing microbial cells; extracting lysed cells with solvent; separating a polar lipid fraction from the extract; and drying the polar lipid fraction, with or without addition of other nutrients, to form a particulate material.

5 11. The method of claim 10, wherein the polar lipid fraction is an aqueous slurry which is dried by spray drying.

12. The method of claim 10, wherein the microbial cells are dinoflagellate cells.

13. The method of claim 10, wherein the microbial cells are cells of
10 *Cryptocodinium cohnii*.

14. An aqueous emulsion or suspension containing phospholipids with docosahexaenoic acid (DHA) residues prepared by homogenizing with water a polar lipid extract from DHA-containing microbes.

15 15. The emulsion or suspension of claim 1, wherein at least 10 % of the fatty acid residues in lipids of the microbes are DHA residues

16. The emulsion or suspension of claim 1, wherein at least 10 % of the fatty acid residues in polar lipids of said microbes are DHA residues.

17. The emulsion or suspension of claim 1, wherein said microbes are dinoflagellates.

20 18. The emulsion or suspension of claim 1, wherein said microbes are *Cryptocodinium cohnii*.

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19. A composition comprising a particulate material containing phospholipids with DHA prepared by drying a slurry comprising a polar lipid extract from DHA-containing microbes and a meal containing protein, carbohydrate, or both.

20. The composition of claim 19, wherein meal comprises microbial cells or
5 cell fragments.

21. The composition of claim 19, wherein the microbial cells or cell fragments are from *Chlorella*.

22. The composition of claim 19 wherein the microbial cells or cell fragments are from *Cryptocodinium*.

10 23. The composition of claim 19, wherein the microbial cells or cell fragments are from a yeast.

24. The composition of claim 19, wherein the microbial cells or cell fragments are from *Mortierella*.

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As the below named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name;

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**DHA-CONTAINING NUTRITIONAL COMPOSITIONS
AND METHODS FOR THEIR PRODUCTION**

the specification of which: ☐ is attached hereto.
☒ was filed on: July 31, 1998
as Application No.: PCT/US98/15835
and was amended on _____ (if applicable).

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56.

Prior Foreign Application(s)

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application Number	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed	
				Yes <input type="checkbox"/>	No <input type="checkbox"/>
				Yes <input type="checkbox"/>	No <input type="checkbox"/>

Prior Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing (day, month, year)
60/054,563	01 August 1997

Prior United States Application(s)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Number	Date of Filing (day, month, year)	Status - Patented, Pending, Abandoned
PCT/US98/15835	31 July 1998	Pending

And I hereby appoint, both jointly and severally, as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys, their registration numbers being listed after their names:

Rodger L. Tate, Registration No. 27,399; Scott F. Partridge, Registration No. 28,142; Jerry W. Mills, Registration No. 23,005; Robert Neuner, Registration No. 24,316; James Remanick, Registration No. 36,902; James B. Arpin, Registration No. 33,470; Laurence H. Posorske, Registration No. 34,666; Floyd S. Chapman, Registration No. 40,555; Robert A. King, Registration No. 42,736; Andrew D. Skale, Registration No. 44,338; Robert L. Troike, Registration No. 24,183; Jay M. Cantor, Registration No. 19,808; Lori D. Stiffler, Registration No. 35,939; and Jay B. Johnson, Registration No. 38,193.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Page 2

** TOTAL PAGE.06 **

Application Number	Date of Filing (day, month, year)	Status - Patented, Pending, Abandoned
PCT/US98/15835	31 July 1998	Pending

And I hereby appoint, both jointly and severally, as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys, their registration numbers being listed after their names:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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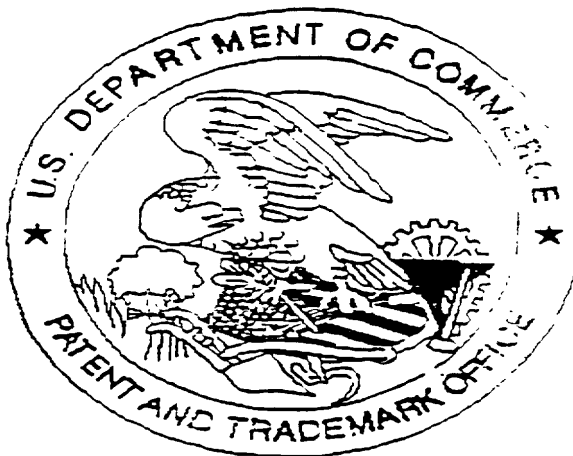
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